Environmental Health Perspectives
Vol. 75, pp. 37-43, 1987

Pulsatile Peptide Secretion: Encoding of Brain Messages Regulating Endocrine and Reproductive Functions

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Neuropeptides are defined chemical messengers produced by the brain to modulate its own activity and also to regulate the function of every organ system. These neuropeptides can be viewed as coded chemical signals produced by the brain and secreted into the blood or into other fluids, such as the cerebrospinal fluid, to be transported and to act at a distant site. The signals arrive to the target organ or sometimes to an intermediary station, such as the pituitary gland, where they are decoded, transformed into a more powerful signal, and sent again through the general circulation to reach their final target. Our work has characterized the episodic or pulsatile pattern of secretion of a number of peptide hormones produced by the brain or the pituitary gland and analyzed the brain mechanisms involved in the generation of such a pulsatile pattern of hormone secretion. Molecular biology approaches have provided information on the synthesis, processing, and secretion of these brain messengers. In addition, using computer-assisted perifusion systems, we have been able to reproduce *in vitro* some of the signals produced by the brain and are currently trying to decode the message carried by those signals, as well as determining the intracellular messengers involved in the signal process. The importance of the neuropeptides and of the messages carried by the pulsatile signal is underlined by experiments in which animals treated with a neurotoxin were rendered infertile. The neurotoxin affects a number of neuronal systems within the brain and destroys or impairs the activity of many peptidergic neurons in areas of the brain related to regulation of reproductive functions. This work has also established that the pulsatile hormone signals seen in normal animals are either absent or grossly impaired in the infertile animals treated with the neurotoxin. Studies of pulsatile hormone secretion are, therefore, very useful peripheral markers for the evaluation of changes in cerebral function. In addition, very valuable diagnostic and therapeutic applications can be derived from the study of pulsatility patterns of neuropeptide secretion.

Introduction

The central nervous system (CNS) regulates the function and activity of every organ system in the body. That regulatory activity is mediated by a very complex series of modulatory mechanisms. Some of these mechanisms involve direct innervation of particular organs, in which case the regulation of function is primarily exerted by the moment-to-moment changes in nerve impulse activity.

Another very important regulatory mechanism involves the endocrine system as a mediator of the interactions between brain and target organ. The brain itself is now recognized as a very important endocrine organ, which, in turn, controls the activity of every peripheral endocrine gland. Through the secretion of hormones into the general circulation or into specialized vascular compartments, the brain can effectively send chemical signals to modify the activity of the different organs or cell systems under its influence. This mechanism of transformation of an electrical impulse into a defined chemical signal is a classical example of signal transduction. As a result of the transduction of the signal, many well-defined substances are secreted into the circulation to reach their peripheral target. The secreted substance can then be viewed as a true messenger carrying an encoded signal to be deciphered only by the specific target system. In recent years, it has become abundantly clear that neural peptides play a major role as brain messengers, modulating a variety of functions. Peptides offer a great degree of flexibility in terms of modifications of the structure of the parent molecule that can occur as a result of processing. Each new peptide product generated may conceivably carry a unique and physiologically relevant message.

With the large number of important functions regulated by the CNS, however, it is difficult to expect that each effect is going to be mediated by a distinct chemical

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messenger. It seems more logical to postulate that a primary messenger is responsible for the regulation of a specific function or set of functions and that changes in the secretory pattern of the primary messenger may elicit secondary events leading to an integrated functional response. This concept requires that the peptide or hormone be secreted in a type of pattern that can be used by the CNS to encode for a series of commands or signals to be decoded by the responsive peripheral cell. Further modulation of the response to the input signal can be achieved by altering the sensitivity of the target cell to the signal, in effect amplifying or dampening the response or even altering not just the magnitude but also the quality of the cellular response.

Over the last decade very compelling evidence has been presented to indicate that most, if not all, hormones are secreted in a pulsatile pattern (*1,2*), and evidence is beginning to emerge to indicate that the pattern of secretion can result in distinct changes in organ function (*1,2*). These concepts have far-reaching implications not only for our understanding of the basic mechanisms governing the input signal/cellular response phenomenon, but also for the clear significance that the pulsatility concept has for diagnostic and therapeutic strategies. Moreover, analysis of abnormal pulsatility patterns of specific neural or pituitary peptides may provide an important biological marker for different neural disorders, including dementias, depression, and many other psychiatric, neurological, or endocrine diseases.

Brain-Pituitary Axis and Primary Chemical Messengers

It has long been recognized that neuroendocrine events are mediated by the interaction of specific neural centers and pathways with an endocrine gland that acts as a transducer and amplifier of the neural signal. This concept is best exemplified by the brain regulation of anterior pituitary function, carried out by a series of specific brain peptides generically described as "hypophysiotrophic hypothalamic hormones," or "hypothalamic releasing or inhibiting factors." Each releasing or inhibiting factor is the prime regulator of the secretion of a pituitary hormone(s), and through that mechanism the peptide hormone can affect a wide range of body functions regulated by a specific endocrine gland.

The diagram shown in Figure 1 describes in a schematic manner the role of three hypothalamic neuropeptides: thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), and corticotropin-releasing factor (CRF), and the cascade of hormones that transduce and modulate the message and ultimately elicit the response or functions regulated by the thyroid, gonad, and adrenal glands, respectively. As clearly indicated in the diagram, a primary messenger (TRH, LHRH, or CRF) activates an intermediate target (pituitary gland). This message from the brain to the pituitary is carried out via a closed-circuit vascular

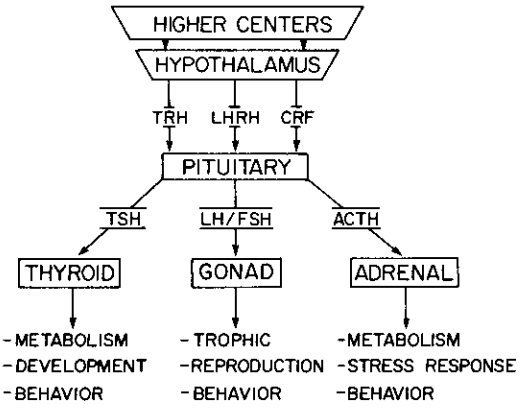

FIGURE 1. Schematic diagram representing the hierarchical organization of the neural peptidergic systems regulating the thyroid, gonad, and adrenal gland. The hypothalamic peptides, thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), and corticotropin-releasing factor (CRF), control the secretion of specific pituitary hormones (TSH, LH-FSH, ACTH), which in turn modulate the activity of the endocrine glands under their control. Ultimately, the hormones secreted from those glands will affect a number of metabolic, developmental, reproductive, and behavioral functions.

bed, the hypophyseal portal blood system, which ensures that the tiny amount of hormone secreted by the brain reaches its target without being diluted and/or metabolized in the general circulation. The intermediate target tissue then generates an amplified signal, i.e., the release of a given pituitary hormone. This more powerful and longer lasting signal can then travel through the general circulation and reach its final target in effective concentrations. The target gland, i.e., thyroid, gonad, or adrenal, responds by secreting additional key hormonal signals, which, in turn, will eventually be responsible for the multiple cellular and functional responses regulated by that particular endocrine organ. It is easy to visualize in this diagram that a "ripple effect" can be obtained by any changes that occur in the signal input via relay stations where signals are decoded, transduced, amplified, and retransmitted with an ever increasing level of complexity and diversification.

Pulsatile Pattern of Hormone Secretion and Neuropeptide Input

As indicated above, most hormones are secreted from their respective cells into the circulation in a pulsatile fashion (*1,2*), and this pattern of secretion is normally controlled by the central nervous system, although some evidence suggests that the isolated organs can also release hormones in a pulsatile manner. The significance of these observations has been clarified by studies that indicate that when secretagogues are infused continuously or in various pulsatile patterns *in vivo*, the response of the target organ can vary dramatically, ranging from normal or even enhanced responses to decreased or suppressed secretion. The relevance of the

pattern of secretagogue administration in dictating target organ response can also be demonstrated in *in vitro* systems designed to evaluate the dynamics of stimulated or inhibited hormone release (*2*).

Evidence from different laboratories, including our own, indicates that as a result of the hypothalamic peptidergic input, all of the anterior pituitary hormones, including LH (*1,3*), FSH (*3,4*), prolactin (*5*), GH (*5,6*), TSH (*7*), and ACTH (*8*), are secreted in a pulsatile or episodic fashion. Some of these pulses have a certain coincidental periodicity, and therefore, at a time when a pulse of a given pituitary hormone is detected, this pulse may coincide with that of other pituitary hormone(s). However, each hormone seems to have a characteristic pulsatile or episodic secretory pattern, and in each case the neuropeptide messenger is the primary regulator of this pattern. Suppression of the secretion and/or of the activity of the primary peptide results in abolition of the pulsatile secretory pattern for the corresponding pituitary hormone (*1–3*).

One effective way of removing the stimulatory peptidergic input is by passive immunoneutralization with highly specific antibodies against the neuropeptide purportedly involved in the regulation of the pulsatile secretory pattern. We have recently employed this approach in order to analyze the role of LHRH in the pulsatile release of LH and FSH (*3*). For this purpose, a potent LHRH antiserum was given to castrated male rats fitted with indwelling right atrial cannula to allow frequent samples (*3*). Sequential 200-μL blood samples were withdrawn every 10 min for 3 hr and replaced with an equal volume of saline. All rats were freely moving and unanesthetized during the collection period. The rats were injected with 500-μL nonimmune serum (NIS) or LHRH antiserum (LHRH-AS) either immediately after withdrawal of the first sample or 24 hr before the initiation of sampling. Previous testing indicated that the binding half-life of the LHRH antiserum after injection in the rat was 24 hr, suggesting that a 500-μL injection would provide maximal immunoneutralization for at least 36 hr. In rats injected with NIS (Fig. 2*A*), plasma LH was secreted in a pulsatile manner. The injection of 500 μL of LHRH-AS immediately after withdrawal of the first sample (Fig. 2*B*) resulted in an abrupt abolishment of pulsatile LH secretion and a rapid reduction of mean plasma LH as compared with NIS injected controls. Pulsatile FSH secretion, as defined by pulse frequency and amplitude, was unaffected by the LHRH-AS throughout the sampling period (*3*). In rats injected with LHRH-AS 24 hr prior to sampling, plasma LH continued to be nonpulsatile and suppressed to the same low level observed after the immediate injection of LHRH-AS (Fig. 2*C*). These results confirm the importance of the LHRH input on the generation of the pulsatile pattern of LH secretion.

An important advantage of immunoneutralizing endogenous LHRH with a specific antiserum is that it allows study of the exogenous replacement of LHRH bioactivity by using a structurally modified LHRH agonist to circumvent the antiserum blockade. The antiserum used for this study (A722) is specific for the 3–10 amino acid sequence of the decapeptide LHRH, including the amidated C-terminus. It is possible, therefore, to bypass the LHRH-AS blockade by using the LHRH agonist [Des-Gly^{10}]-LHRH ethyl amide (DG-LHRH), which possesses 2.7 times the releasing activity of LHRH, but which crossreacts less than 0.01% with A772. Using this approach, LH pulsatility can be reestablished by giving the agonist at selected intervals in bolus injections. The effect of varying regimes of administration on the LH pulsatile pattern and also on the LHRH-dependent and LHRH-independent regulation of pulsatile and basal FSH secretion can also be evaluated (*9*).

The Impact of Pulsatile Hormone Secretion on Target Tissue Response and Function

An excellent example of the impact that the pattern of stimulation can have on the function of a system has been provided by work on the role of exogenously administered LHRH on pituitary gonadotropin secretion

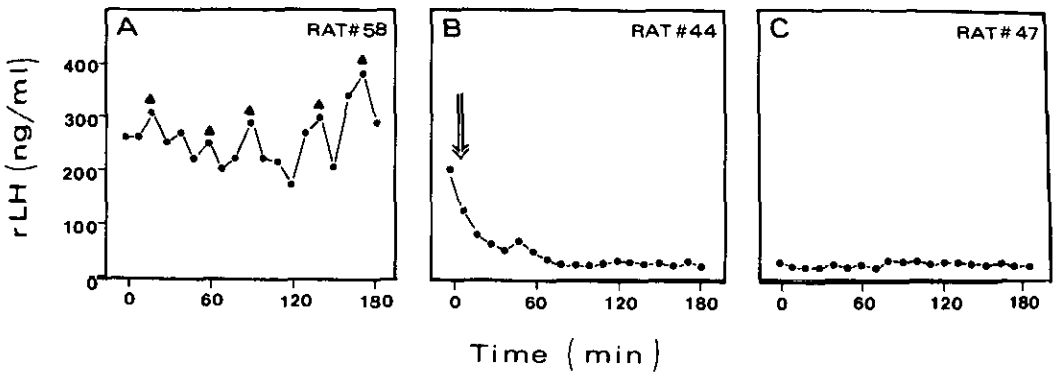

FIGURE 2. (*A*) Pattern of pulsatile LH secretion in an untreated, castrated male rat and evidence for the dependency of LH pulsatile pattern on the input of the neural peptide LHRH. (*B*) Arrow indicates the time of immunoneutralization with a potent LHRH antiserum given IV. Note the immediate drop in serum LH levels and the absence of LH pulses. (*C*) The effects of the antibody persist for at least 24 hr.

patterns in rhesus monkeys, whose endogenous input signal (LHRH) had been suppressed by mediobasal hypothalamic lesions (*1*). In such animals, LH and FSH secretion fall to very low or undetectable levels but can be reestablished by the pulsatile administration of LHRH. If the pulsatile administration of LHRH is replaced by a continuous infusion, however, an inhibition of gonadotropin secretion is observed, which continues throughout the infusion period. When the pulsatile mode of LHRH administration is reestablished, secretion of both gonadotropins increases dramatically. Additional studies have demonstrated that the type of stimulation pattern (pulsatile versus constant) is important in determining the gonadotropin release response and the characteristics of the pulsatile pattern. Changing the frequency of the LHRH pulses can subsequently alter the release of LH and FSH. Decreasing the frequency of the LHRH pulses from 1 per hour to 1 pulse every 3 hr reduced LH secretion markedly while producing a modest increase in FSH release, resulting in an increase in the plasma FSH/LH ratio. Restoring the frequency to 1 pulse per hour returns both hormones to normal plasma levels and ratio (*1*). Conversely, increasing the pulse frequency from 1 LHRH pulse per hour to 2, 3, or 5 pulses per hour produces an increasing suppression of the release of both gonadotropins, which is restored to control levels by resumption of the original 1 pulse per hour pattern of administration. It has also been demonstrated that, after a certain level, the absolute amount of peptide infused contributes very little to the observed response (*1*). The pattern of administration is by far the most relevant component of the input signal.

Variations in the Pattern of Signal Input and Generation of Cellular Responses *In Vitro*

Studies *in vitro* also support the importance of the input signal configuration for receptor activation (or inactivation) and for response modulation. Dispersed, anterior pituitary cells cultured for 3 to 4 days in the presence of a given secretagogue and then stimulated with the same substance show a suppressed response when compared to the response of cells cultured in medium alone. A specific example can be observed in a study from our laboratory illustrated in Figure 3. Rat anterior pituitary cells cultured for 4 days in control medium release significant amounts of LH in a concentration-related manner when exposed to LHRH for a 3-hr incubation period (Fig. 3*A*). Cell cultures exposed continuously to LHRH during the last 3 days of culture show a significantly blunted response to LHRH on day 4 (Fig. 3*B*), indicating that some form of desensitization occurs as a consequence of the protracted stimulation. When the same stimulus is applied for 2 days of culture followed by a 24-hr rest period without exposure to LHRH, the cells partially recover their sensitivity and respond to LHRH in a concentration-dependent manner

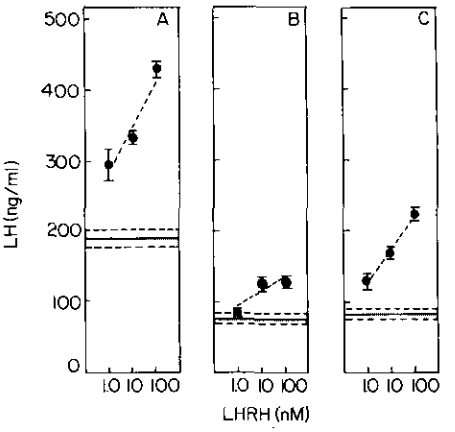

FIGURE 3. Desensitization of the gonadotrope response to LHRH *in vitro*. (*A*) Dispersed anterior pituitary cells were cultured for 3 days and then exposed for 3 hr to increasing concentrations of LHRH, which elicited a dose-dependent increase in LH secretion. (*B*) If the cells are continuously exposed for 3 days to LHRH, the basal secretion (horizontal bar and shaded area representing SE) of the cells is decreased and the response to LHRH during the 3-hr test is severely blunted. (*C*) When the continuous exposure period is restricted to 2 days followed by a 24-hr recovery period, a partial recovery of the response can then be observed.

(Fig. 3*C*). Similar observations have been reported for somatostatin inhibition of GH secretion (*10*). If, however, somatostatin is administered to the cells in a pulsatile fashion, desensitization fails to occur, demonstrating that just as in the *in vivo* model, pulsatile input delivery is required to maintain target cell responsiveness.

Application of Computerized Perifusion to the Study of Signal Configuration, Signal Transduction, and Intracellular Messengers Linked to the Secretory Response

Although the importance of pulsatile stimulation is now well recognized, very little is known about how the characteristics of a pulsatile signal affect the final target cell response, and practically nothing is known about the intracellular pathways that respond to and/or require a pulsatile stimulation. The major reason for this lack of information has been the limitation of conventional perifusion systems in producing a pulsatile signal. For this reason, as well as for convenience and methodological logistics, most studies of intracellular regulators of LH secretion have relied on static incubation or cultured cell models in which the cells receive a constant exposure to the stimulatory substance. Recently, however, a computer-assisted perifusion apparatus was developed that can control and/or manipulate all parameters of a pulsatile hormone signal. Using this system, we have designed a LHRH pulse that rises from zero to peak concentration in 30 sec and then decays exponentially, according to the perifusion flow rate (*2,11*).

When perifused hemipituitaries taken from intact male rats are exposed to this LHRH signal design, the result is a LH secretory response that closely mimics the shape of LH pulses that are observed *in vivo* (*2,3*). Through experimentation to determine the proper pattern (frequency and amplitude) of pulse administration, we have found that LHRH pulses of 10 ng total mass, delivered once each 40 min, can maintain the responsiveness of the perifused hemipituitaries (*2*). Under these conditions, the first two pulses of LHRH delivered consistently stimulate less LH secretion than the subsequent pulses. This may represent a priming action of LHRH in which the pituitary initially must be sensitized to the peptide before establishing a stable pattern of release.

We have utilized this system to study the intracellular messengers mediating LH secretion, in response to a dynamic pulsatile LHRH stimulation, by designing an experimental model in which perifused hemipituitaries from intact male rats are exposed to three 10-ng total mass priming pulses of LHRH followed by a 50-ng total mass LHRH pulse of similar design. After 100 min, to allow time for the resultant rise in LH secretion to return to baseline levels, a second, 50-ng total mass pulse is delivered either alone or concomitantly with a constant infusion of a given drug or inhibitor known to affect a specific intracellular messenger system. The drug/inhibitor infusion is initiated 30 min prior to the generation of the second pulse. The dynamics of LH secretion, in response to the 50-ng LHRH pulses, are examined in detail by collecting 30-sec fractions of perfusate. A typical LH secretory response to this regimen, in the absence of any intracellular messenger-affecting drug, is illustrated in Figure 4. Both the LHRH signal and the LH response were measured in the perifusate fractions by radioimmunoassay. The priming effect of the three 10-ng LHRH pulses, which serve to sensitize and stabilize the LH secretory response in preparation for the later large pulses, can clearly be observed. The frequent sampling procedure revealed a sharp rise in LH secretion which would reach 300% baseline within 10 min and which was evident within the first fraction in which the LHRH signal was detected (Fig. 5). Although the LHRH pulse was completely washed out of the perifusion chamber within 9 min of initiation, the LH secretory response was greatly protracted, lasting 70 to 80 min before fully returning to baseline levels (Fig. 5). During the gradual decay of the LH response to baseline levels, LH appeared to be secreted in episodic waves (*11*) (Figs. 4 and 5).

The LH secretory response to the second 50-ng total mass pulse (approximately 1.72 μg rLH) was not significantly different in magnitude or shape as compared with the LH response to the first 50-ng LHRH pulse (approximately 1.65 μg rLH). Trials were also conducted in which the hemipituitaries were perifused during the same time frame and with the same fraction collection intervals but were exposed either to only the three, 10-ng LHRH priming pulses (no large LHRH pulses) or to medium alone (no LHRH). In both instances, LH secretion, during the time intervals in which the 50-ng LHRH pulses would have normally been delivered, was characterized by a stable baseline pattern of secretion (*12*).

Additional studies were designed to analyze the role of specific intracellular messenger systems known to be involved in the secretion of LH on the pulsatile pattern of LH, again using the computerized perifusion system. The receptor-activated enzyme, phospholipase C, liberates two putative intracellular messengers, inositol triphosphate, which initiates mobilization of intracellular Ca^{2+}, and diacylglycerol, which activates protein kinase C. As a requirement for a substance to be considered as an intracellular mediator of an extracellular primary messenger, in this instance LHRH, the putative intracellular messenger should be able to induce the same cellular response as the primary messenger. Computer-assisted perifusion, in combination with the described model for observing secretion dynamics, is an ideal system to examine this question because of the ability to visualize both the magnitude and shape of the responses.

To compare the LH secretory response induced by phospholipase C with that induced by the 50-ng LHRH pulses, a similarly designed pulse of phospholipase C was delivered to perifused hemipituitaries after the three 10-ng total mass priming pulses of LHRH. The pulse of phospholipase C stimulated a rapid rise in LH secretion similar to that induced by LHRH in both time sequence and shape. Like the LHRH-induced LH secretory response, the gradual decay of the LH response to phospholipase C was composed of small episodic waves of LH secretion, which continued long past the calculated washout time of the phospholipase C pulse (*12*). The similarities between the LHRH- and phospholipase C-induced LH secretory pattern suggest that LHRH may act through a phospholipase C-mediated intracellular system.

Ionic Ca^{2+} is intimately involved in the LH secretory response generated by individual pulses of either LHRH or phospholipase C. Indeed, in the presence of the Ca^{2+} chelator EGTA, the secretory LH response was essentially abolished, confirming the belief that the availability of free ionic Ca^{2+} is an essential requirement for the actions of these secretagogues.

Many studies from different laboratories, including our own (*13*), suggest that key arachidonic acid metabolites play an important role in the transduction of the signal generated by certain classes of hormones. Within the pituitary, the release of LH induced by LHRH has been found to be partly dependent on the release of arachidonic acid and the subsequent generation of arachidonate metabolites via the lipoxygenase pathway. In our system, addition of the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA) prior to the LHRH stimulatory pulse, blunted the LH secretory response, with an effective 50% reduction in the total mass of hormone being secreted (*12*). These results suggest that the arachidonate metabolites participating in the LH secretory response are primarily involved in the amplification of the response to the input signal, rather

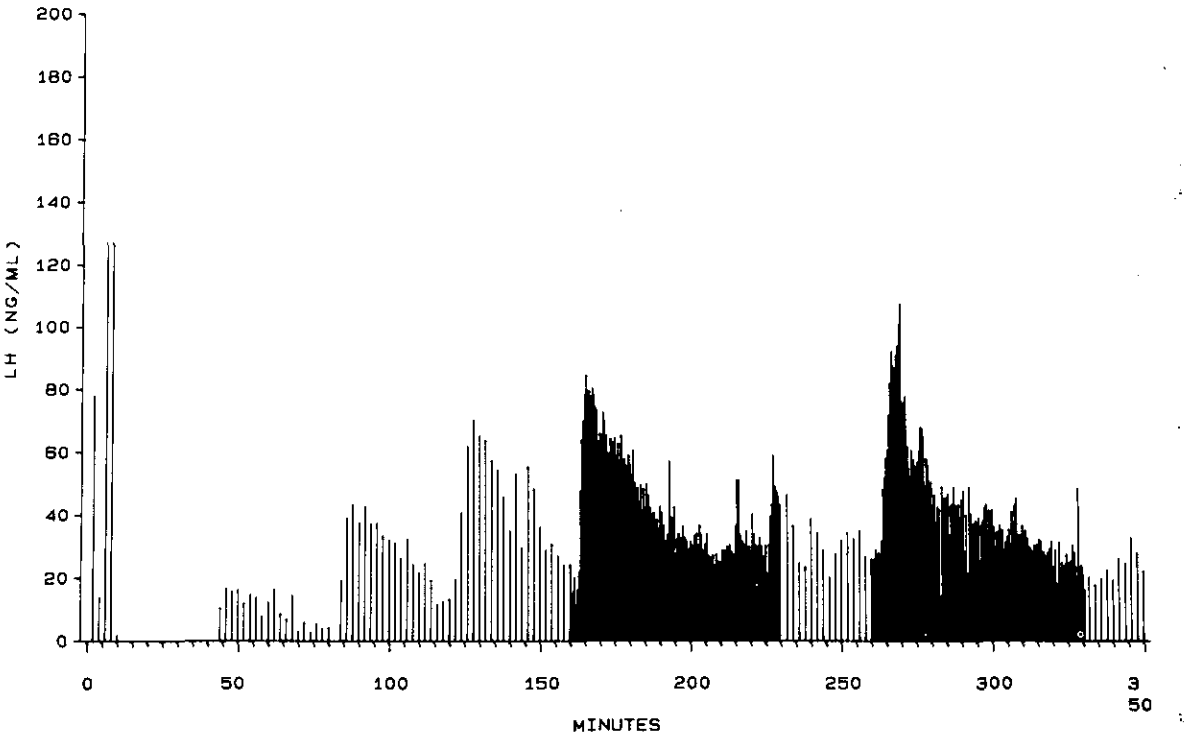

FIGURE 4. *In vitro* LH secretory pattern from a hemipituitary perifused with a computerized-perifusion system, according to the experimental design described in the text. Each vertical line represents the amount of LH secreted per collection interval. Reproduced with permission (*13*).

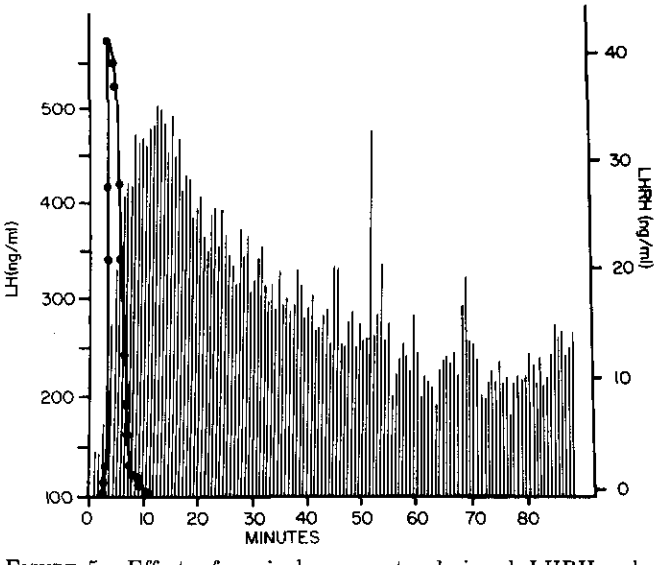

FIGURE 5. Effect of a single, computer-designed LHRH pulse (heavy line, dark circles) of 50-ng total mass on LH secretion from a hemipituitary using computer-assisted perifusion. The LHRH pulse was delivered after three 10-ng total mass priming LHRH pulses, as shown in Fig. 4. LH and LHRH were measured by radioimmunoassay in 0.5-min perifusate fractions. Note the episodic secretory events superimposed on the decaying phase of the secretory response. Baseline secretion in this chamber was at about 100 ng LH/mL before the LHRH pulse. Reproduced with permission (*13*).

than on modifying the characteristics (shape, timing, etc.) of the response.

Stimulus and Secretion: Decoding the Input Signal

The *in vitro* data obtained from our computerized perifusion studies clearly indicate the complexity of the interactions mediating the cellular response to a controlled, pulsatile input. Indeed, we have recently obtained evidence that the delivery of a peptide (LHRH) from the hypothalamus to the pituitary through the hypophyseal portal vasculature occurs also in a pulsatile fashion (Ching, Valenca, and Negro-Vilar, unpublished). The characteristics of each individual pulse vary from pulse to pulse and from animal to animal. The variations are primarily in shape and magnitude, frequency being a relatively more constant parameter. Nevertheless, certain parameters can be averaged and consistent trends can be observed from animal to animal. Changes in some parameters of pulsatility occur as a consequence of experimental manipulations. It is tempting to speculate, therefore, that the signals carry a number of distinct messages related not only to the transmission of a command to increase hormone secretion, but perhaps to convey other messages which would result in more subtle changes in the qualitative, dynamic, and integrative aspects of the secretory response. Our ability to decode these messages will

largely depend on two main conditions: (a) establishing a valid, flexible input signal-cellular response *in vitro* system which can be modulated to closely mimic *in vivo* conditions, and (b) expanding our analysis capabilities to measure the secretory response (hormone secretion) to evaluate not only quantitatively the secretory process but also to be able to monitor qualitative (molecular forms of hormone secreted, bio- and immunoactivity, etc.), dynamic (pattern and configuration of secretory episodes), and integrative aspects, including other secretory events (i.e., cosecretion of other molecules) that may occur as a result of a given input signal.

We have made good progress in establishing an *in vitro* system that meets many of the needs as stated in (a). Initial studies seem to provide some support and information to some of the aspects described in (b). However, a thorough analysis of all the different aspects of the secretory event is going to require a concerted and multifaceted approach in order to unravel the intrinsic coding of information that may govern the neural control of the endocrine system.

We thank Sandra Sandberg for her excellent secretarial and editorial assistance.

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